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# Up-regulation of p53 by antisense expression of HPV18 E6 oncogene does not influence the level of MN/CA IX tumor-associated protein in HeLa cervical carcinoma cells

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**Abstract.** Oncogenic potential of human papillomaviruses is related to capacity of HPV-encoded oncoproteins to bind and inactivate tumor suppressor proteins. Interaction of p53 with HPV E6 results in aberrant regulation of various cellular genes. We evaluated the possible involvement of *MN/CA9* gene, whose expression is closely associated with cervical carcinomas, in regulatory pathways driven by p53 and E6. We demonstrated that one of the two p53 consensus sequences present in *MN/CA9* promoter participates in DNA-protein interaction but it does not bind p53. Tetracycline-inducible antisense expression of HPV18 E6 in human cervical carcinoma HeLa cells resulted in increased level of p53 but did not affect expression of MN/CA IX protein. Therefore we conclude that at least in HeLa cells there is no direct relationship between expression of MN/CA IX and expression of E6 or p53.

## Introduction

Human papillomaviruses (HPV) include at present more than 80 distinct genotypes that are divided into high- and low-risk groups according to their association with the development of malignancies. HPV-16, HPV-18 and several additional high-risk types have been detected in at least 90% of cervical carcinomas and more than 50% of other anogenital cancers (1,2). Transforming properties of HPVs are connected to expression of E6 and E7 genes whose products are growth-stimulating proteins (3). E6 and E7 are both necessary and sufficient for the immortalization of primary human squamous epithelial cells (4,5) and their continuous expression is required for the maintenance of the malignant phenotype of HPV-transformed C4-I cervical cancer cells (6).

The E6 gene encodes a small acidic protein of approximately 150 amino acids that forms two zinc fingers each consisting of two Cys-X-X-Cys motifs (7). The most significant features of the E6 protein, related to its oncogenic potential, are the capacity to bind tumor suppressor protein p53, induce its degradation via the ubiquitin-dependent proteolysis and inhibit p53-mediated transcriptional activation as well as repression (8-11).

p53 plays a fundamental role in regulation of cell proliferation and control of the genome integrity (12). It can act as a transcriptional regulator by virtue of its ability to bind specific DNA sequences or sequester transcription factors (13-18). Functional inactivation of p53 leading to cell transformation can be caused by certain mutations or by association with oncoproteins of DNA tumor viruses (19). Abrogation of the tumor suppressing function of p53 induced by HPV E6 appears to be instrumental in HPV-mediated neoplastic progression. Correspondingly, cervical cell lines expressing HPV E6 possess wild-type p53, while in cell lines devoid of HPV E6 the p53 gene undergoes inactivating mutation (20).

Protein MN/CA IX is a novel member of carbonic anhydrase family encoded by a single copy gene whose sequence and structure have been described (21,22). It was originally detected as a plasma membrane antigen in the human cervical carcinoma cell line HeLa and in a number of human carcinomas but not in the corresponding normal tissues (23,24). MN/CA IX expression *in vitro* was found to correlate both with the density of HeLa cells and with the tumorigenic phenotype of CGL1 and CGL3 hybrids between HeLa and normal human fibroblasts (23,24). Introduction of *MN/CA9* cDNA into NIH 3T3 cells resulted in their morphological transformation and increased proliferation (21). Immunohistochemical and PCR analyses of tumor tissue samples revealed association of MN/CA IX mainly with carcinomas of cervix uteri, kidney, bladder, esophagus and colon (25-30). More than 90% of dysplastic or malignant tissues of cervix uteri showed immunoreactivity for the MN/CA IX antigen (25). On this basis, Liao and Stanbridge

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(30) have proposed the utility of MN/CA IX as an early biomarker of cervical neoplasia.

Association of both HPVs and MN/CA IX protein with cervical carcinomas raised questions about their possible relationship. In addition, promoter region of *MN/CA9* gene was found to contain two p53-binding sites indicating that p53 could represent a link between HPVs and MN/CA IX.

This work was performed to elucidate the proposed relationship between expression of MN/CA IX, p53 and HPV18 E6 in human cervical carcinoma cell line HeLa. We have employed antisense expression of E6 cDNA under control of tetracycline inducible system. We detected increased level of p53, while the expression of MN/CA IX protein remained unchanged. Thus, our investigation did not find any evident relationship of HPV E6 and p53 to MN/CA IX in HeLa cells.

## Materials and methods

*Construction of antisense vector.* Entire HPV18 genome cloned in pBR322 (from Dr S. Hallez, Free University of Brussels) was linearized with PstI and used as a template for amplification of E6 by PCR. Following primers were used to generate the 495 bp E6 cDNA: P1 5'-CCGGAATTCA TGGCGCGCTTTGAGG-AT-3' and P2 5'-ATTCCGC GGTTATACTTGTGTTTCTCTGA-3'. Sequence of obtained PCR product was verified by T7 sequencing kit (Pharmacia Biotech) after cloning into *Eco*RI and *Sac*II sites of pBluescript SK+ (Stratagene). After digestion with *Eco*RI and *Sac*II the E6 cDNA was cloned in an antisense orientation into expression vector pUHD10-3 (31).

*Cell culture and transfection.* HeLa tTA cell line (Clontech, C3005-1) was maintained in DMEM medium (BioWhittaker) supplemented with 10% FCS (Gibco BRL), gentamycin (80 μg/ml, LEK) and geneticin (100 μg/ml, Gibco BRL) in the presence of 5% $CO_2$ at 37°C. The cells were plated in a 60 mm dish at a density of $5x10^5$ 24 h before transfection. At 80% confluence, they were transfected with 13.5 μg of pUHD10-3 antiE6 and 1.5 μg pTK-Hygro (Clontech) using calcium phosphate transfection method (profection mammalian transfection system, Promega). 48 h after transfection, the cells were trypsinized, plated on 10 dishes and selected in DMEM with 10% FCS, hygromycin B (100 μg/ml, Clontech) and tetracycline (2 μg/ml, Serva).

*Preparation of nuclear extracts.* CGL1, CGL3, HeLa and MCF-7 cells were cultivated in DMEM and 10% FCS. MCF-7 cells were treated at subconfluence with 5 μg/ml mitomycin C (Sigma) in order to induce p53 and harvested for nuclear extract preparation 16 h after treatment (32). All nuclear extracts were prepared as described by Willems *et al* (33). Protein concentrations were determined with a BCA kit (Pierce).

*DNase I footprinting assay.* DNA fragment of *MN/CA9* promoter corresponding to region -243/+31 with respect to transcription start was $^{32}$P-end-labeled by Klenow fragment and digested to produce single strand labeling. Purified DNA fragments were incubated with nuclear extracts from CGL1 and CGL3 cells. DNase I footprinting was performed with SureTrack Footprinting (Pharmacia). Samples were analyzed on 8% sequencing gels.

*DNA binding assay.* Following oligonucleotides were used: CONp53 primer containing consensus sequence for wild-type p53 (CONp53) 5'-GAAATTCTCGAGGAACATGTCC CAACATGTTGCTCGAG-3' and its complement 5'-GCTC GAGCAACATGTTGGGACATGTTCCTCGAGAATT-3' (kindly provided by Dr K. Pitkänen, Haartman Institute, Helsinki), PR1 sequence from protected region of MN promoter from position -46 to -26 5'-AGGCTTGCTCCTCC CCCACCCAG-3' and its complement 5'-GCTGGGTGGGG GAGGAGCAAG-3', PR1mut, i.e. form of PR1 with mutation in AP-2 binding sequence 5'-CCAGGCTTGCTCCTCCCTT ACCCAG-3' and its complement 5'-GCTGGGTAAGGGAG GAGCAAGCCTG-3'. Oligonucleotides were annealed and radiolabelled using T4 polynucleotide kinase (Gibco BRL) and γ-$^{32}$P-ATP (3000 Ci/mmol, Amersham Life Sciences). Probes were purified in QIA-Gene columns (Qiagene). Binding reactions (20 μl ~4 μg of nuclear extracts) were performed in 20 mM HEPES-KOH pH 7.9, 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 0.5 mM DTT, 0.025% NP-40, 2 mM spermidine, 1.5 μg poly dI-dC and incubated with approximately 80 fmol of the labeled probe for 20 min at room temperature. In some experiments, 500 ng of monoclonal antibody PAb421 against p53 (kindly provided by Dr B. Vojtesek, Masaryk Memorial Cancer Institute, Brno, Czech Republic) and in some cases excess of cold probe for CONp53 and PR1 were added. For supershift experiments, p53-specific monoclonal antibody DO1 (gift of Dr B. Vojtesek) was used. Samples were applied on a non-denaturing 4.5% polyacrylamide gel with 5% glycerol in 0.3X TBE. After 1 h pre-run, electrophoresis was carried out at 300 V for 3 h at 4°C. Gels were dried and autoradiographed.

*Preparation of cell lysates.* Hygromycin-resistant clones were cultivated in the presence and absence of tetracycline, respectively, in 35-mm dishes. After 72 h, cells were extracted with 50 mM Tris-HCl, 250 mM NaCl, 15 mM $MgCl_2$, 2 mM EGTA, 1 mM PMSF, 1% Triton X-100 pH 7.5 during 30 min at 0°C. Protein concentrations were determined with a BCA kit (Pierce).

*Western blot analysis.* Samples containing equal amounts of total proteins were electrophoretically separated on 10% SDS-PAGE and subsequently transferred to nitrocellulose membrane (Hybond ECL, Amersham Life Sciences). Membranes were blocked in TBS (50 mM Tris-HCl, 150 mM NaCl pH 7.5) containing 5% non-fat milk for 1 h, incubated with 1 μg/ml anti-p53 monoclonal antibody DO1 (34) and MN/CA IX-specific monoclonal antibody M75 (23). Membranes were washed three times with TBS, incubated with horseradish peroxidase-conjugated swine anti-mouse antibodies and subjected to detection by ECL method (Amersham Life Sciences).

## Results

Computer analysis of *MN/CA9* promoter sequence has revealed the presence of several *cis*-regulatory elements, including

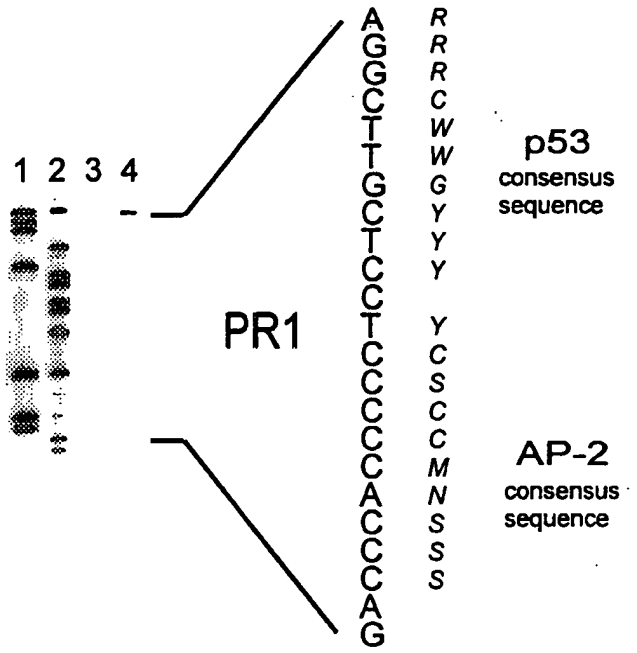


Figure 1. DNase I footprinting analysis. DNA fragment of *MN/CA9* promoter corresponding to region -243/+31 was incubated with 40 µg of nuclear extracts prepared from CGL1 cells (lane 3) or CGL3 cells (lane 4). Lane 1, Control reaction with BSA instead of nuclear extract. Lane 2, Maxam-Gilbert A+G sequencing reaction of the DNA fragment that was used for the analysis. Sequence of PR1 protected region (-46/-26) obtained with both CGL1 and CGL3 nuclear extracts is shown.

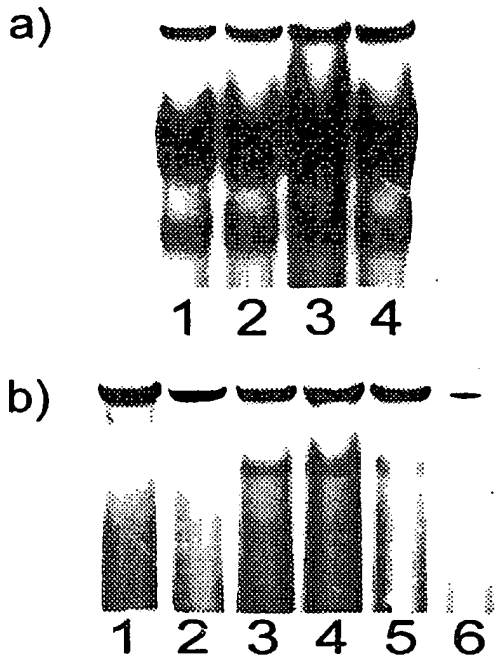


Figure 2. Analysis of protein binding to PR1 (a) and CONp53 (b) oligonucleotides. a, 80 fmol of labeled ds oligonucleotides containing sequence of PR1 region of *MN/CA9* promoter (-46/-26) were incubated with nuclear extract (NE) prepared from mitomycin C-treated MCF-7 cells as follows: Lane 1, 4 µg of NE only; lane 2, 4 µg of NE, 500 ng of PAb421; lane 3, 9 µg of NE, 1 µg of PAb421; lane 4, 4 µg of NE, 500 ng of PAb421, 150-fold molar excess of unlabeled oligonucleotide CONp53. Mobility shift pattern was similar in all variants of experiment suggesting absence of p53-specific complex. b, 80 fmol of labeled ds oligonucleotides CONp53 (containing consensus sequence for wild-type p53) were incubated with: Lane 1, 4 µg of NE from mitomycin treated MCF-7 cells, 500 ng of PAb421 and 1 µg of DO-1; lane 2, 4 µg of NE only; lane 3, 4 µg of NE, 500 ng of PAb421; lane 4, 9 µg of NE, 1 µg of PAb421; lane 5, 4 µg of NE, 500 ng of PAb421 and 150-fold molar excess of unlabeled oligonucleotide PR1; lane 6, 4 µg of NE, 500 ng of PAb421 and 150-fold molar excess of unlabeled oligonucleotide CONp53.

two p53 binding sites. The first site was at position -46/-37 with respect to the transcription start, the second one was at position -214/-205 (22). This fact raised the possibility that p53 might be involved in transcriptional regulation of *MN/CA9* gene.

We first determined whether the p53-related *cis*-regulatory elements in *MN/CA9* promoter could participate in DNA-protein interactions *in vitro*. DNase I footprinting (FTP) assay of relevant promoter region (-243/+31) was carried out using nuclear extracts prepared from both the CGL1 non-tumorigenic hybrid cells that do not express MN/CA IX as well as from the CGL3 tumorigenic cells expressing MN/CA IX (24). The p53 consensus sequence that is more distant from the transcription start is not protected in DNase I FTP (data not shown). However, the promoter region -46/-26 designated PR1 (5'-AGGCTTGCTC CTCCCCCACCCAG-3') was clearly protected at both DNA strands (Fig. 1). In addition to the putative p53 binding site (5'-AGGCTTGCTC-3'), this region contains a consensus sequence for AP-2 transcription factor (5'-TCCCCCACCC-3'). Protection pattern of PR1 did not show any differences in binding of nuclear proteins from MN/CA IX-positive CGL3 cells versus negative CGL1 cells.

Gel mobility shift assay was utilized to analyze binding of p53 to the binding site in PR1. As a source of wild-type p53 MCF-7 human mammary carcinoma cells treated with mitomycin C were used (32). Incubation of nuclear extract prepared from these cells with labeled double stranded oligonucleotide derived from PR1 resulted in formation of three DNA-protein complexes (Fig. 2a). Presence of p53 and AP-2 consensus sequences in PR1 indicated that these complexes might contain corresponding proteins. However, the mobility shift pattern was the same irrespective of the presence or absence of monoclonal antibody PAb421 that induces DNA-binding activity of p53 *in vitro* (32). Moreover, addition of competing unlabeled CONp53 oligonucleotide containing p53 consensus sequence did not interfere with the formation of these complexes, suggesting that p53 was not involved in binding to tested DNA. This assumption was verified by a supershift experiment incubating PR1 first with the PAb421 and then with the monoclonal antibody DO1, which binds to the N terminus of human p53 (34). This experiment did not reveal any supershift (data not shown). In contrast, employment of CONp53 oligonucleotide as a positive control resulted in formation of both p53-specific mobility shift and supershift (Fig. 2b). In accord with previous data, unlabeled PR1 oligonucleotide added in 150-fold molar excess was not able to compete for p53 binding. These results indicated that the

experimental conditions for gel shift assay were correct and that under these conditions, p53 is unable to recognize and bind the p53-like regulatory element in *MN/CA9* promoter.

On the other hand, participation of AP-2 transcription factor in two of three complexes with PR1 was proved by inhibition of shifting activity by 50-fold molar excess of unlabeled commercial oligonucleotide with AP-2 consensus sequence (data not shown). Since the binding sites for p53 and AP-2 are in close vicinity in PR1, it was not inconceivable that AP-2 moiety could spherically inhibit interaction of p53 with its target DNA sequence. To abolish the binding of AP-2, we used PR1-mut oligonucleotide CCCC/TC/TAACC with mutations in AP-2 consensus sequence. As expected, gel shift assay with PR1-mut led to decreased binding of AP-2 and at the same time to increased binding of an unknown protein (Fig. 3). This unknown protein probably binds to the region of PR1 that contains the putative p53 binding site. Gel shift experiment using PR1-mut, nuclear extract from MCF-7 and PAb421 did not lead to detection of p53. Our results suggest, that the p53-like binding sequence present in *MN/CA9* promoter does not come into contact with p53, but appears to interact with a protein whose identity is presently unknown.

However, evidence against binding of p53 to *MN/CA9* promoter did not exclude its involvement in regulation of *MN/CA9* gene expression. It is known that p53 can act as a transcriptional repressor of a wide variety of promoters without contacting the DNA but via binding the proteins required for basal transcription (12,15). Based on this knowledge, we analyzed the effect of increased p53 expression on the level of MN/CA IX protein. As a model, we used human cervical carcinoma cells HeLa that express MN/CA IX protein, contain HPV18 genome and possess low amount of wild-type p53 as a consequence of degradation by E6 viral oncoprotein.

We employed tetracycline-inducible expression system composed of tetracycline-regulated expression vector pUHD-10-3 and HeLa tTA cells that stably harbor a gene encoding tetracycline (TTC) repressor (31). Expression of the foreign gene inserted into the pUHD10-3 vector and introduced to HeLa tTA cells is turned off in the presence of TTC and released in its absence. HPV18 E6 cDNA of 495 bp was prepared by PCR and cloned in an antisense orientation into pUHD 10-3 vector. The obtained construct was co-transfected with pTK-hygro into HeLa tTA cells. The transfected cells were selected by hygromycine for two weeks and cloned. Ninety hygromycine-resistant clones were tested for expression of p53. This strategy of indirect detection of E6 antisense expression was based on the known negative correlation between levels of E6 oncoprotein and p53 tumor suppressor protein. The transfectants were cultured for 3 days in the presence and absence of TTC, respectively. Cellular extracts from paired samples were analyzed by Western blotting using human p53-specific monoclonal antibody DO1. We obtained seven clonal cell lines that showed several fold-increased levels of p53 protein following TTC withdrawal (Fig. 4a). In the course of incubation of these clones, the cells showed only slight decrease in proliferation in absence of TTC as analyzed by MTS-based cell proliferation kit (Promega), and the resulting density was comparable to density of TTC-treated cells (data not shown). The blots analyzed first for


Figure 3. Mobility shift assay of PR1 and PR1mut oligonucleotides derived from the protected region -46/-26 of the *MN/CA9* promoter. Reactions contained 4 µg of nuclear proteins extracted from HeLa cells (lanes 1 and 2) and from mitomycin C-activated MCF-7 cells (lanes 3 and 4), 80 fmol of PR1 (lane 1) or PR1mut containing mutations in AP-2 binding sequence (lanes 2, 3 and 4) and 500 ng of PAb 421 (lane 4).

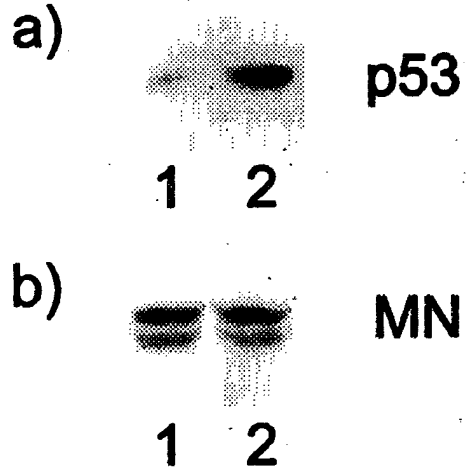


Figure 4. Western blotting analysis of p53 protein and MN/CA IX protein levels in representative clone of HeLa tTA cells expressing antisense E6. HeLa tTA cells were co-transfected with plasmid pUHD10-3 containing antisense E6 cDNA and pTK-Hygro, selected, subcloned and cultured in the presence (lane 1) and absence (lane 2) of tetracycline for 3 days. Cellular extracts were analyzed by immunoblotting using p53-specific monoclonal antibody DO-1 (a) and MN/CA IX-specific monoclonal antibody M75 (b).

p53 were reprobed with M75 for expression of MN/CA IX protein. This analysis did not show any differences at the level of MN/CA IX protein in TTC-treated versus untreated cells (Fig. 4b). Thus, our results suggest that up-regulation of p53 does not have any effect on MN/CA IX expression and that there is no evident relationship between expression of HPV 18 E6 oncoprotein and MN/CA IX protein in HeLa cells.

## Discussion

Infections by high-risk human papillomaviruses have been closely linked to the development of cervical carcinomas based on numerous clinical data and *in vitro* transformation experiments in various models (2,3). Introduction of high-risk HPV genomes into primary human epithelial cells results in their immortalization, but is not sufficient for their full malignant transformation (35-37). This fact suggests existence of cellular factors contributing to cervical carcinogenesis. MN/CA IX protein is a candidate for such cellular factor since its expression has been associated with a very high percentage of cervical neoplasia (25,30). These data prompted us to investigate the relationship between HPV and MN/CA IX. Moreover, presence of two putative p53-binding sites in *MN/CA9* promoter (22) directed our attention to possible involvement of p53 tumor suppressor protein in this relationship.

Our experiments have shown that only one of the two p53 consensus sites of *MN/CA9* promoter region PR1 participates in DNA-protein interaction, but we failed to prove p53 binding to this site even after abrogation of AP-2 interaction with the neighboring sequence. This observation corresponds with the described requirement for two closely localized p53 regulatory elements for efficient recognition and trans-activation by p53 (38,39). However, we found that the p53 consensus sequence binds another protein *in vitro* and this binding sequence is occupied both in cells expressing and non-expressing MN/CA IX. Since there are no reports on protein(s) other than p53 that could interact with p53 consensus sequence, the identity of the protein binding to PR1 cannot be anticipated and remaines to be determined.

In the case of direct contact of p53 to DNA, p53 was shown to function as a transcriptional activator of numerous growth-suppressing genes (13,14). On the other hand, trans-repressive activity of p53 does not require direct interaction with DNA, but is dependent on protein-protein interactions with transcriptional factors. In this way, p53 negatively regulates transcription of genes encoding growth-stimulating proteins (12). Thus, the possible involvement of MN/CA IX in carcinogenesis would not deny an idea of repression by p53.

Based on the above, we followed consequences of increased p53 level on MN/CA IX protein expression in HeLa cells. In these HPV 18-infected cells, wild-type p53 is subjected to inactivation via degradation triggered by E6 viral oncoprotein (9). As a result, level of p53 in HeLa cells is low (40), but is inducible by treatment with genotoxic drugs (41). Such treatment, however, exerts pleiotropic effects and therefore it was not suitable for our purposes. Alternatively, level of p53 could be increased by its ectopic expression from strong heterologous promoter, but the amount of produced p53 would be far from the physiological values and the experiment could provide artificial data. Therefore, we have chosen an experimental system employing tetracycline-controlled antisense expression of HPV E6 oncogene that allowed us to target specifically E6 and to regulate level of p53 within physiological limits. We have shown that antisense expression of E6 resulted in several fold-increased level of p53 but did not affect the expression of MN/CA IX protein.

These results do not provide any evidence in favor of relationship between either HPV E6 or p53 to MN/CA IX, but rather suggest that at least in human cervical carcinoma HeLa cells, MN/CA IX protein expression is independent of regulatory events mediated by E6 and p53. Similar conclusion can be drawn from an additional study in which HPV E7 antisense expression in HeLa cells did not influence the expression of MN/CA IX protein (unpublished data).

The possible relationship between HPV status and MN/CA IX protein expression was addressed in two previous reports (42,43). Resnick and co-workers (42) studied expression of MN/CA IX and distribution and type of HPV in cervical epithelial neoplasia. They found that the proportion of neoplastic cells immunoreactive for MN/CA IX protein did not correlate with HPV types grouped by their association with cancer. Moreover, MN/CA IX antigen was not expressed in a pattern that consistently paralleled HPV replication. According to Brewer *et al* (43), low expression of MN/CA IX protein in tissue specimens of cervical neoplasia correlated with HPV negativity.

In agreement with our observations, results of these clinical studies support the lack of direct relationship between MN/CA IX and HPV status. The available data altogether suggest that HPV infection and MN/CA IX protein expression are independent events in the multistep process of cervical carcinogenesis.

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